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2-23-07
Date

Signature

In re Application of:

André DELACOURTE
Nicolas SERGEANT

Serial No.: 10/625,854

Filed: July 23, 2003

For: PREVENTION, TREATMENT AND DIAGNOSIS OF DISEASES ASSOCIATED WITH BETA-AMYLOID FORMATION AND/OR AGGREGATION

Group Art Unit: 1649

Examiner: Chang Yu Wang

Atty. Dkt. No.: 11362.0039.NPUS01

Confirmation No.: 9442

RULE 132 DECLARATION OF DR. EUGEEN VANMECHELEN

My name is Dr. Eugeen Vanmechelen. A copy of my current Curriculum Vitae is attached as Appendix 1. I hereby declare as follows:

1. I am employed by Innogenetics NV, the assignee of United States Patent Application Serial No. 10/625,854 (the "Application").

2. My position at Innogenetics NV is Principal Scientist for the neurodegenerative disease branch of Innogenetics' Diagnostic division. I have been employed by Innogenetics since March 1987.

3. I am familiar with the technology described in the Application because Innogenetics has a longstanding interest in biological markers of Alzheimer's disease and has been successful in the development of cerebrospinal fluid biomarkers for Alzheimer's disease. Along with other scientists at Innogenetics, I have collaborated with the named inventors to develop

this disclosed technology following their initial discovery. My involvement is evidenced by my co-authorship on the first peer-reviewed publication regarding the newly-disclosed subject matter in the Application (*See* Sergeant et al., 2003 (Ref. 5 herein)).

4. As a result of the discovery and the collaboration, I (in conjunction with scientists other than the Application's named inventors) have recently used several monoclonal antibodies specific for different Aβ epitopes in a multiparameter approach to demonstrate that the relative levels of N-truncated Aβ are increased in very early stages of Alzheimer's disease in living patients. Specifically, in stages described as Mild Cognitive Impairment (MCI) or MCI developing into Alzheimer's Disease (MCI-AD) (*See* Vanderstichele et al. (2005) (Ref. 6 herein)).

5. I have reviewed the August 23, 2006 Office Action regarding the Application.

6. I understand that the Examiner has rejected certain claims in the Application directed to N-terminal truncated β-Amyloid variants for lack of enablement, in part, because: "The data shown in Figures 4 and 7 and Table 8 indicate that N-terminally truncated Aβ peptides 8-42 and 5-42 are detected in the CSF of AD patients. However, Aβ8-42 is also detected in the S0 control and so are Aβ11-42 and Aβ10-42, indicating that the presence of these forms of N-terminal truncated Aβ is a natural phenomenon." (Office Action, p. 5).

7. I have been instructed that for a U.S. patent claim to be enabled, the Application must enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the claimed invention.

8. The patients in Figures 4 and 7 and (and Table 6) in the Specification were from the geriatric department of E. Roux Hospital who were hospitalized for various reasons. All patients received a full examination, including a standard neurological examination.

Cognitive status was evaluated using the Mini-Mental State Examination (MMSE) and the Clinical Dementing Rating (CDR). As described in Delacourte et al. (1999) (Ref. 2 herein), these tests were performed every six months by the same investigator. PHF-tau pathology was assessed in each of the subjects after the patients died.

9. The newly-disclosed results in the Application regarding the presence and identification of N-terminal Aβ peptides are an extension of the results reported in the attached Delacourte et al. (1999) and Delacourte et al (2002) publications (Refs. 2 and 3 herein, respectively), which were directed to assessing PHF-tau pathology.

10. Because the data reported in Figures 4 and 7 and Table 6 of the Application is an extension of the work reported in the 1999 and 2002 Delacourte et al. studies, the term "control" is used differently in Figures 4 and 7 and Table 6 of the Application, than as the term is defined in on page 4 of the Application. (Figures 2-4 and 7 (with legends), Tables, 3 and 6-8, and page 4 of the Specification are attached for the convenience as "Appendix 2"). As carried over in the Delacourte et al. (2002) publication, the designation of "control" was assigned to the patients cohort in the 1999 study based on: (i) a clinical assessment of the living patient as summarized in paragraph 8 of this declaration (from the description in the Delacourte et al. (1999) and (2002) publications), and (ii) the observable progression of PHF-tau pathology in specified regions of the patients' brains after they died as defined in Delacourte et al. (1999). In other words, the presence or absence of β-Amyloid did not influence the designation of a subject as a "control" in the prior studies; rather, the designation was made based, in physical part, on a postmortem assessment of **tau pathology.**

11. In Figures 4 and 7 and Table 6, subjects designated as "controls" refer to a mixture of subjects that either underwent a normal aging process in the absence of tau pathology (S0), or those that have tau pathology (S1-S7) but are either clinically non-demented or in their preclinical stages of AD (*See* Delacourte et al. (1999)).

12. The alternative definition of the term "control" on page 4 of the Specification was also adapted from the Delacourte et al. (1999) and Delacourte et al. (2002) publications. This alternative definition of a "control," however, was based on the authors' proposed "Criteria to Establish a Biochemical Diagnosis of AD (CEBDAD)." (See Delacourte et al. (1999) at p. 9, and Delacourte et al (2002) at p. 405) The proposed criteria were formulated following the examination of subjects classified based on progression of PHF-tau pathology (as discussed in paragraph 10 above) and on the concentration of β-amyloid in brain tissue determined after death. On page 4 of the Specification, the term "control" is used to assess subjects that: (i) were non-demented while living; (ii) have no detectable $A\beta_{42}$ aggregates in neocortical areas of the brain; and (iii) have limited or no presentation of PHF-tau pathology (based on the scale presented in Delacourte et al. (1999)).

13. Neither the Delacourte et al. (1999) publication, nor the Delacourte et al. (2002) publication discusses the early detection or identification of N-terminal truncated or post-translationally modified $A\beta_{42}$ peptides as said peptides are described in the Application. One key aspect of the Application is the detection and identification of N-terminal truncated forms of $A\beta_{42}$ in early stages of Alzheimer's pathology. Further, as described in the Delacourte et al (2002) publication, the tau pathology staging and cortical β-amyloid staging performed in the 1999 and 2002 Delacourte et al. publications were performed only on dead subjects.

14. As described in Figures 4 and 7, a subject designated as "S0" is one that has no PHF-tau protein in any part of the brain, including amygdala and basal nucleus of Meynert. These nondemented subjects were <73 years old, and no amyloid deposits or neurofibrillary tangles were detectable in any area of their brains (*See* Delacourte et al. (1999)). Thus, an S0 subject is one that falls within the definition of "control" discussed in paragraph 10 above (*i.e.*, one with no observable tau pathology).

15. Figure 4 of the Application shows detection of the pool of $A\beta_{42}$ extracted from the brain tissue of the respective subjects. No $A\beta_{42}$ peptide was detected in the particular subject investigated in that study classified as S0. Alternatively, $A\beta_{42}$ peptide was increasingly detected in subjects with more advanced neurofibrillary degeneration (NFD) as defined in Delacourte et al. (1999). The subjects with more advanced NFD are designated by an increased "S-score" (e.g., S10) which is assigned based on the appearance of PHF-tau in different regions of the brain.

16. The only "gold standard" for AD diagnosis is a neuropathological examination of the brain for the number of tangles and plaques in particular brain regions. This neuropathological analysis, however, can only be accomplished after the subject has died. Further, the biochemical pathway of neurofibrillary degeneration (NFD) or PHF-tau pathology is believed to reflect the natural history of Alzheimer's disease in the brain. Those clinically-diagnosed patients with advanced NFD demonstrate typical Alzheimer's Disease (AD) pathology. Part of typical AD pathology is the formation of amyloid plaques resulting from amyloidosis. (*See* Delacourte et al. (2002)). The absence of $A\beta_{42}$ peptides in some S0 subjects (*i.e.*, those subjects with no tau pathology) (see, for example, Fig. 4 and Fig. 7 ("S0 (Duc)") in the Application) demonstrates that $A\beta_{42}$ is not a "naturally occurring"

constituent of normal brain tissue. Alternatively, detection of the N-terminal truncated $A\beta_{42}$ peptides in patients with tau pathology (with and without clinical dementia) indicates that it is a marker that is related to Alzheimer's disease etiology (See Delacourte et al. (2002)).

17. Figure 7 of the Application shows detection of N-terminally truncated $A\beta_{42}$ peptides in a variety of subjects, including some that are designated as "controls" in Table 6. (Table 6 describes results from experiments performed on tissue of the same deceased subjects). Detection of N-terminally truncated $A\beta_{42}$ peptides in patients designated as "controls" (*i.e.*, patients with tau pathology but no clinical impairment or dementia) is significant because the detection of $A\beta_{42}$ peptides in preclinical stages of AD (as assessed by tau pathology) provides an independent, early evaluation of progressing Alzheimer's pathology.

18. As described in the Application, Table 8 shows detection of Aβ 5-42, Aβ 8-42, Aβ 10-42 and Aβ 11-42 in cerebrospinal fluid (CSF) from in a variety of living patients that comprise a different patient cohort. As used in the left column of the table, the heading "Group" refers to a clinical diagnosis (*See* Vanderstichele et al. (2005)) (Ref. 6 herein) of the patient population based on parameters described in Table 7 and the accompanying text, including: Age, MMSE score, tau concentration, Phospho-tau concentration, and Aβ1-42 concentration. Tau concentration, Phospho-tau concentration and Aβ1-42 concentration in these subjects was determined from the respective patient's CSF samples obtained via lumbar puncture.

19. Detection of the indicated N-terminal truncated $A\beta_{42}$ peptides in some of the "control" patients of Table 8 (e.g., "control" Nr 148) is significant because such patients might be in either the preclinical or infraclinical stages of Alzheimer's disease; that is, where a combination of tau pathology and clinical assessment may not signal the subject's risk or

susceptibility of Alzheimer's Disease. Also, detection of N-terminal truncated species in CSF may signal the early stage of β-Amyloid pathology.

20. As shown in Figures 4 and 7 and Table 8, detection of Aβ peptides 8-42, 10-42, and 11-42 in patients designated "S0" and "Control" is not a natural phenomenon because, as described above, there exist two populations of patients, both of which are clinically defined "control" based on tau pathology—one population that is defined as normal aging with no tau pathology, and one that has preclinical or infraclinical AD (as determined by tau pathology after death). Living patients with preclinical or infraclinical AD may benefit from detection of N-terminal-truncated Aβ peptides when a clinical evaluation does not reveal AD-induced abnormalities.

21. As detailed above, it is my opinion that a person skilled in the art would understand that, as described in the Application, the detection of N-terminal truncated β-Amyloid variants or post-translationally modified β-Amyloid variants in living patients with either, preclinical or infraclinical, or symptomatic Alzheimer's disease is useful for assessing risk or susceptibility to the neurodegenerative disease. Further, detection of N-terminal-truncated Aβ peptides in deceased subjects designated as "S0" or "control" (after death) is not merely indicative of a natural phenomenon; rather, detection of said peptides may provide an alternative means for staging Alzheimer's disease postmortem.

22. In further support of my description above, I have attached additional data marked as Appendix 3.

23. The data in Appendix 3 is related to the data disclosed in the current application because these experiments are an extension of the previous observations in which the $A\beta_{42}$ profile has not only been defined in terms of tau pathology (S0-S10 stages), but also in terms of

Aβ pathology (also defined as stages S0-S10) (*See* Deramecourt et al. (2006) (Ref. 4 herein).

24. I am familiar with the experiments and the results shown in Appendix 3 because we have performed these experiments in close collaboration with Delacourte's research group as evidenced by our co-authorship in the recently-published Deramecourt et al. (2006) paper.

25. In Table 1 included in Appendix 3, "Clinical Diagnosis" refers to established clinical criteria as described in Delacourte et al. (1999) and Delacourte et al. (2002). The heading labeled "Cortical Biochemical Beta-amyloid staging" refers to staging as recently described in Delacourte et al. (2002) and Deramecourt et al. (2006) (Ref. 4 herein). Further the heading "Cortical Biochemical PHF-tau staging" refers to the Tau pathology staging as described in Delacourte et al. (1999). As experiments with these subjects are ongoing, the data for PHF-tau staging is not complete. The most current collection of data is presented in the Table.

26. As shown in Appendix 3, no N-terminal truncated Aβ peptides were detected in the "Control" patients highlighted in yellow.

27. Alternatively, Aβ 1-42, 4-42, and 8-42 peptides were detected in other "Control" patients.

28. In Appendix 3, the "Control" patients highlighted in yellow are different from those "Control" patients whose samples contained Aβ42 peptides because the yellow-highlighted subjects represent normal aging, whereas the other controls are most likely in their very early stages of AD.

29. As shown in Appendix 3, the Aβ 1-42, 4-42, and 8-42 peptides appear before other N-terminally truncated Aβ42 peptides in preclinical stages of AD. The early detection of

these particular peptides is also shown on Figures 2-4 and 7, and pages 51 to 55 of the Application. The findings in these pages of the Application have recently confirmed by several other investigators (Nicoll et al. (2006); Piccini et al. (2005); Patton et al. (2006)).

30. As shown in Appendix 3, no Aβ42 peptides were detected in the "control" patients highlighted in yellow. Each of the designated $A\beta_{42}$ species were detected in at least some MCI, FAD, and AD patients, however, in a manner directly correlated to the increase in the Cortical Biochemical Beta-amyloid staging of the tested patient population. The increasingly frequent detection of N-terminal truncated $A\beta_{42}$ peptides in progressing neurodegenerative disease is significant because its close association with the natural history of AD makes it an ideal candidate for a biological marker.

31. The Application identifies N-terminal truncated $A\beta_{42}$ peptides in preclinical AD patients (those without dementia), confirmed by both biochemical and pathological examination. Identification of the specific N-terminal truncated $A\beta_{42}$ peptides in early stages of β-Amyloid pathology is critical for assessing living patients' risk or susceptibility to Alzheimer's disease because these forms of $A\beta_{42}$ are thought to initiate the formation of amyloid plaques.

32. The bottom panel of Figure 2 (See Appendix 2) shows the presence of different forms of the N-terminal truncated peptides from a subject with full blown AD as detected by Western analysis. The identity of each N-terminal truncated β-Amyloid variant as determined from mass spectrometry is shown in Table 3. For example, "spot 9" (corresponding to a pI of 6.3) in the bottom panel of Figure 2 is identified as comprised of the Aβ4-42, Aβ5-42 and post-translationally modified Aβ4-42 via mass spectrophotometry in Table 3. Figure 3 of the Application shows the result of a similar analysis performed on

the brain of a subject with infraclinical AD. Again, "spot 9" is observed in samples obtained from infraclinical AD patients. This comparison shows that Aβ4-42 and Aβ5-42 are, in fact, one of the primary N-terminal truncated species observed in subjects with early stages of Alzheimer's pathology.

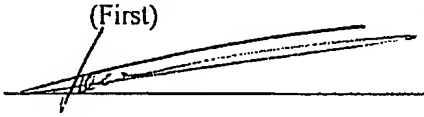
33. As demonstrated in Figures 2 and 3 of the Application (See Appendix 2 herein), the results were confirmed by similar experiments using a transgenic mouse models in Casas et al. (2004) (Ref. 1 herein). Using transgenic mice susceptible to AD-like pathology, the authors (which included the named inventors) showed in Figure 3 of the article that N-terminal truncated forms Aβ4-42 and Aβ4/5-42 (pI 6.3) and Aβ4/5-42 through Aβ8/9/10/11-42 were the first components found in amyloid plaques relating to amyloid pathology. The measurements in Figure 3 of Casas et al. (2004) were taken over time, simulating the progression of AD pathology. The Casas et al. (2004) experiments were conducted in a manner similar to those described in Figures 2 and 3 of the Application as evidenced by the citation to Sergeant et al. (2003), which reproduces the experiments described in the Application.

34. In my opinion, a person of ordinary skill in the art would recognize that detection of any of the described N-terminal truncated Aβ42 species in the Application may be useful for assessing risk or susceptibility to Alzheimer's disease in a living patient. Further, the techniques described in Table 8 for detecting and identifying N-terminal truncated $A\beta_{42}$ peptides in CSF of living patients has recently been used to confirm the increased presence of certain N-terminal truncated $A\beta_{42}$ peptides in CSF of Moderate Cognitively Impaired living patients that later develop AD. (*See* Vanderstichele et al. (2005)).

35. I declare that all statements made herein of my own knowledge are true and correct, and that all statements made on information and belief are believed to be true, and further that I have made these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity and/or enforceability of this application or any patent that may issue thereon.

As a person signing below:

Full Name: Eugeen (First) (Initial) Vanmechelen (Last)

Signature: 

Date: 22 feb 2007. Country of Citizenship: Belgium

Residence Address: Ten Edestraat 101, 9810 Nazareth-Eke, Belgium
(Include number, street name, city, state, and country)

Post Office Address: _____
(if different from residence address) _____

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APPENDIX 1

March, 2006

CURRICULUM VITAE

Vanmechelen Eugeen, Ph.D.

Personal:

Born:	12 August 1959, Turnhout, Belgium
Marital Status:	Married, Catherine Vandevoorde
Children:	Gaelle, Yannick, Gaétan
Office Address:	INNOGENETICS NV R&D department, Diagnostics Industriepark Zwijnaarde 7 box 4 9052 Gent-Zwijnaarde Belgium Tel: +32 9 2410711; Fax: + 32 9 2410907 E-mail: eugeen_vanmechelen@innogenetics.com
Home Address:	Ten Edestraat 101 9810 Nazareth-Eke Tel: +32 9 3856398

Education:

1977:	Koninklijk Atheneum, Mol, Belgium
1981:	Licentiaat Biologie, groep dierkunde, biochemie with distinction, University of Gent, Belgium
1986:	Ph.D., Molecular Biology, Prof Fiers, University of Gent, Belgium

Professional

1987:	Employee Innogenetics, Gent

Teaching Experience

Several undergraduate and graduate (doctoral level) students from 1988 till present

Invited Lectures/Seminars

Several national and international presentations.

Publications:

First and co-author in several book chapters.

Patents:

First and co-author on several patent applications.

Peer Reviewed Journal Papers:

1. Andreasen N, **Vanmechelen E**, Van D, V, Davidsson P, Hesse C, Tarvonen S, Raiha I, Sourander L, Winblad B, Blennow K (1998) Cerebrospinal fluid tau protein as a biochemical marker for Alzheimer's disease: a community based follow up study. J Neurol Neurosurg Psychiatry 64: 298-305.

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APPENDIX 2

FIGURE LEGENDS

Figure 1. Partial amino acid sequence of APP770, displaying the amino acid sequence of Aβ with the α-, β-, and γ-secretase cleavage sites indicated.

Figure 2. Two-dimensional electrophoretic analysis of Aβ species present in brain obtained from Alzheimer's disease patients. Aβ aggregates solubilized with formic acid were resolved by 2-D gel electrophoresis. Aβ monomers (4 kDa) and dimers (8 kDa) were labelled with antibodies WO2 and 6E10 against the amino-terminal region of Aβ (N-ter(5-8) and N-ter(4-13) panels, respectively). The carboxy-terminal tails of $A\beta_{42}$ and $A\beta_{40}$ were detected with 21F12 and ADA40, respectively (Aβ-42 and Aβ-40 panels). The major Aβ species recovered with our extraction method were stained with Coomassie Blue, and 10 spots were subsequently analyzed by mass spectrometry (Table 3). The results presented were obtained from the AD patient showing the largest quantity of amyloid deposits. The isoelectric points (pI) and the Aβ spots used for mass spectrometric analysis are indicated. Note that dimeric species of Aβ are not stained by Coomassie Blue.

Figure 3. Aggregated Aβ species present in brain obtained from infraclinical AD patients. Formic acid solubilized Aβ species derived from the brain tissue of non-demented patients were resolved by 2-D gel electrophoresis. $A\beta_{40}$ species are not detected with our ADA40 antiserum. 21F12 detects both $A\beta_{42}$ monomers (4 kDa staining) and dimers (8 kDa staining). Both WO2 (panel N-ter (5-8)) and 6E10 (panel N-ter (4-13)) antibodies stained a single Aβ peptide spot with pI 5.30.

Figure 4. Dimers of Aβ in infraclinical AD are essentially composed of amino-truncated Aβ peptides. The brain tissue of a control individual (S0), three non-demented cases (S1, S2, and S6) and one AD case (S10) were lysed in formic acid. According to the nomenclatures defined by Delacourte et al. (2002) and Braak and Braak (1991), the stages of tau pathology (S0 to S10) and the amyloid staging classification (B or C) are indicated at the top of the lanes, respectively. Ten ng of Aβ peptides 1-40 and 1-42 were loaded in parallel (first and second lane). Aβ x-42 was

identified with 21F12 (panel Aβ-42) and the amino-terminal region with WO2 (WO2 panel). Molecular weights are indicated on the left, and an arrow indicates the amino-truncated variants labelled by 21F12. Note that the same AD case was used for both 2-D electrophoretic analysis and mass spectrometry.

Figure 5. Principle of the bridging assay as used in Example 2.

Figure 6. Analysis of the specifity of the antibodies by use of the bridging assay. The different N-terminally truncated amyloid peptides were used for coating, and specific HRP-peptide conjugates were used for detection. The antibodies were generated as described in Example 2.

Figure 7. Analysis of the N-terminally truncated Aβ peptides present in human brain extract. OD450 values are shown for a 1/20 dilution of a formic acid extract of several stages of Alzheimer pathology. Peptides were captured on a plate with 21F12 (plate from kit K-1080, Innogenetics, Ghent, Belgium) and detected with biotinylated 3D6 for 1-42, and by a bridging assay for the different antisera specific for truncated amyloid.

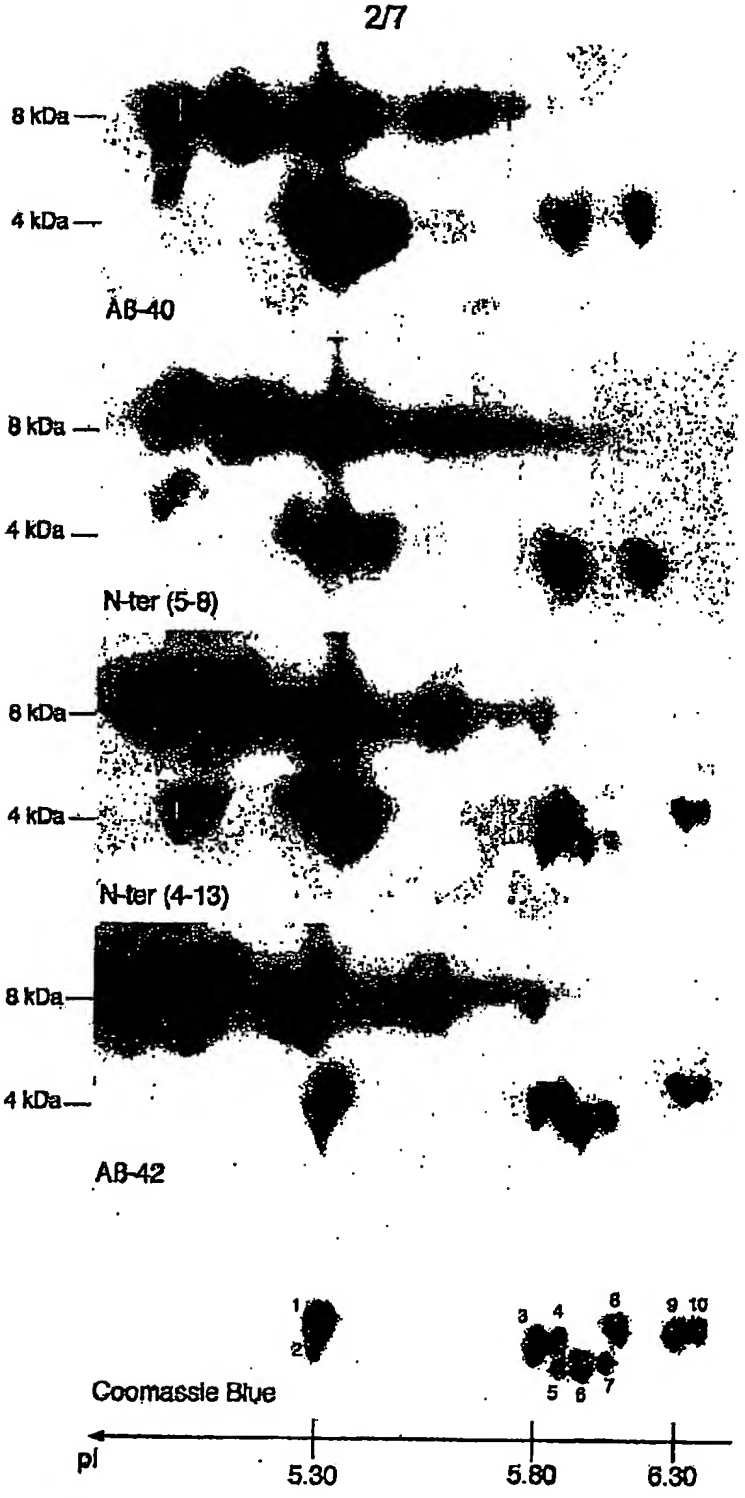
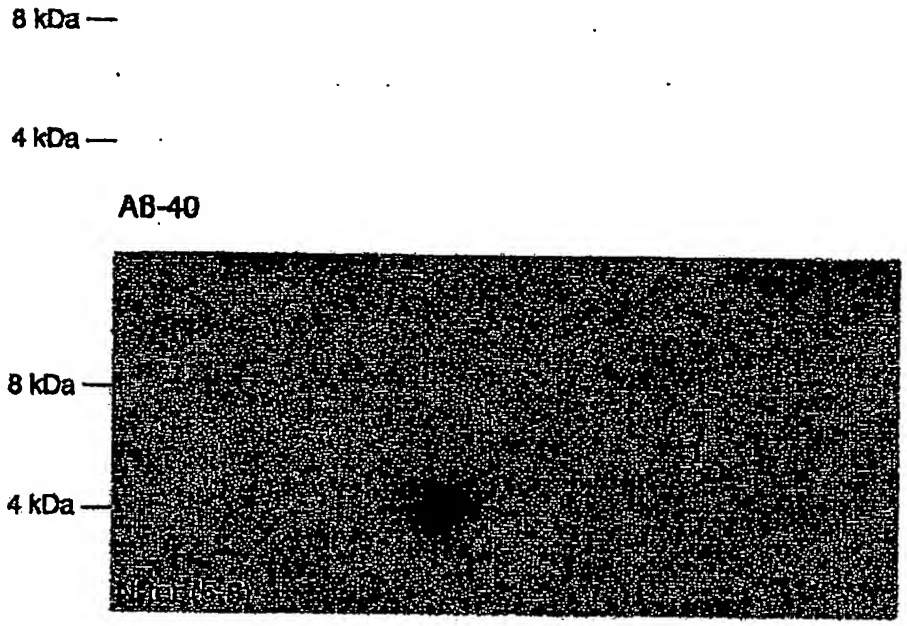
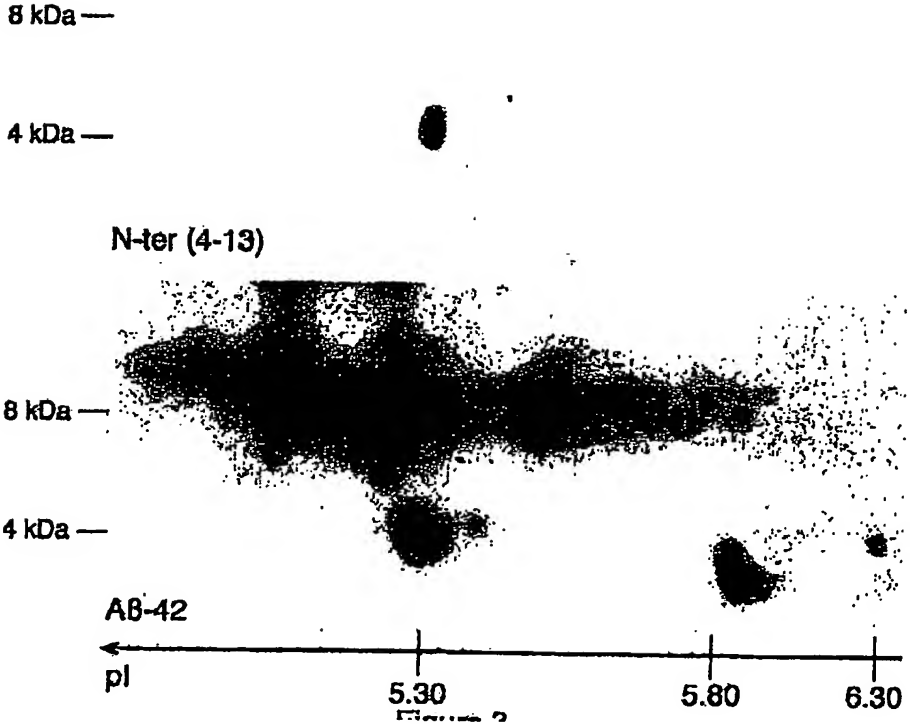

8 kDa
4 kDa
Aß-40
8 kDa
4 kDa
N-ter (5-8)
8 kDa
4 kDa
N-ter (4-13)
8 kDa
4 kDa
Aß-42
1
2
3
4
5
6
7
8
9
10
Coomassie Blue
pI
5.30
5.80
6.30

Figure 2


8 kDa
4 kDa
Aß-40
8 kDa
4 kDa


8 kDa
4 kDa
N-ter (4-13)
8 kDa
4 kDa
Aß-42
pI
5.30
5.80
6.30

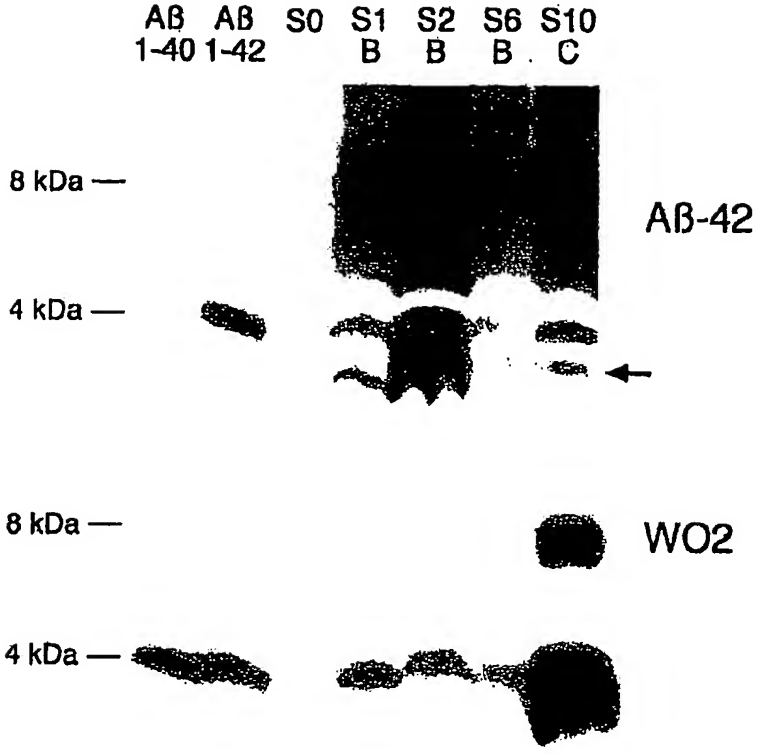


Figure 4

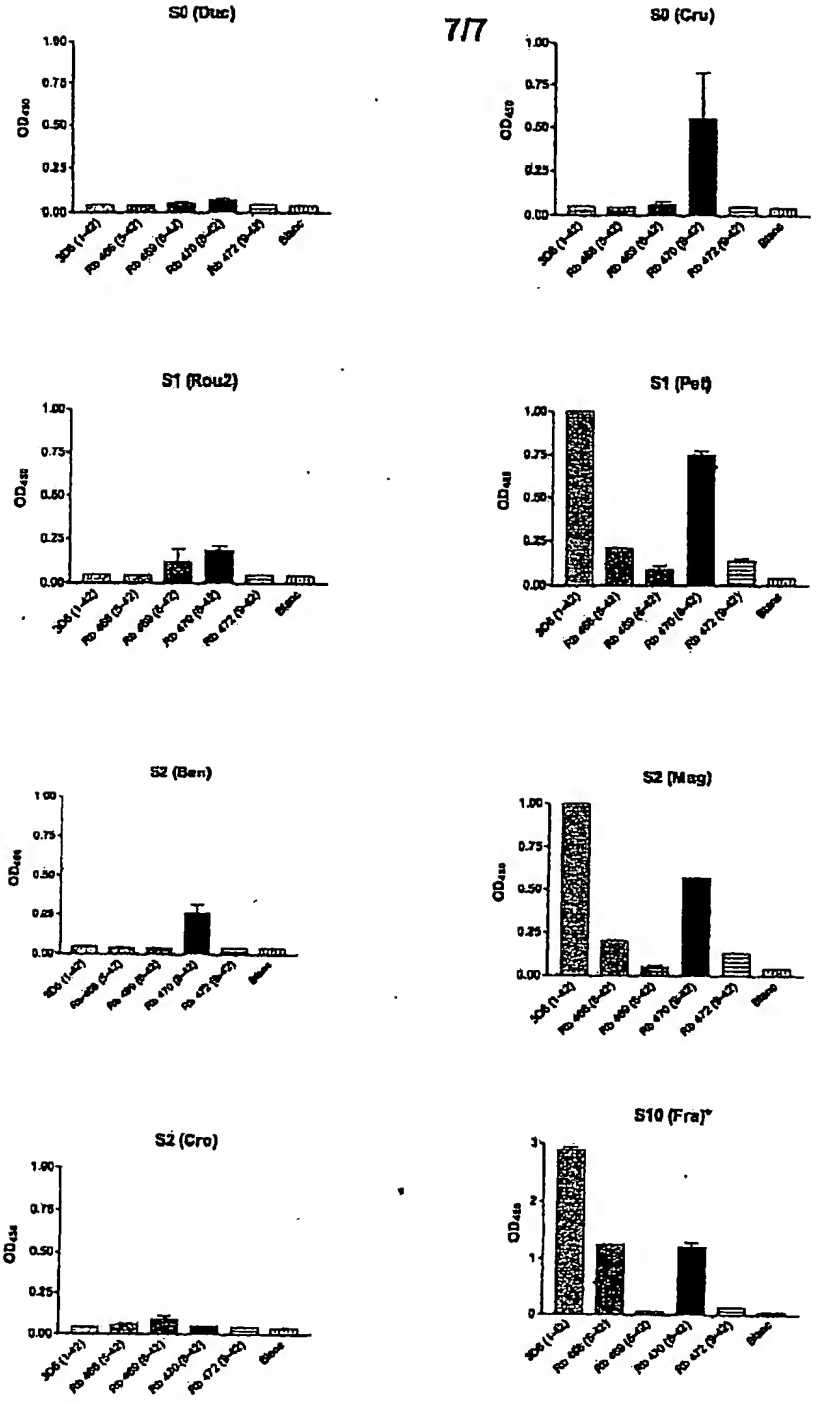


Figure 7

Table 3. Digested peptides of Aβ variants present in Alzheimer's disease patients

Spot	Aß proposed identity[1]	Theoretical mass	Observed mass	Relative amount[2]	Theoretical pI	Observed pI
1	1-16	1954.879	1954.875	23%	5.31	5.3
	1-16+CH_3	1968.905	1968.863	-	5.31	5.3
2	1-16	1954.879	1954.875	10%	5.31	5.3
	1-16+CH_3	1968.905	1968.863	-	5.31	5.3
3	2-16	1839.852	1839.851	6%	5.78	5.8
	2-16+CH_3	1853.878	1853.854	-	5.78	5.8
	(3-16)	1768.815	1768.804	-	5.78	5.8
4	pyrE 3-16	1751.784	1750.790	10%	6.27	5.9
	(2-16)	1839.852	1839.833	-	5.78	5.9
5	pyrE 3-16	1751.784	1750.881	8%	6.27	6.3
6	8-16	1084.517	1084.557	10%	5.96	6.0
	9-16	997.485	998.525	-	6.01	6.0
7	8-16	1084.517	1084.518	13%	5.96	6.1
	9-16	997.485	997.477	-	6.01	6.1
	10-16	940.463	940.460	-	6.01	6.1
9&10	4-16	1639.772	1639.848	16%	6.27	6.3
	4-16+CH_3	1653.798	1653.859	-	6.27	6.3
	5-16	1492.704	1492.770	-		

[1]Methylated fragments are indicated with a CH_3. PyrE corresponds to a pyroglutamyl residue at the N-terminus of the identified fragment. The peptide corresponding to amino acid sequence 17-28 of Aβ was found in all spots (not shown).

[2]The relative amount corresponds to the quantification using Melanie III software on Coomassie stained gels.

Table 6. Analysis of the amount of Aβ(1-42) and N-terminally truncated Aβ in formic acid brain extracts obtained from different cases (control, infraclinical stages 1 and 2, and end-stage AD). Clinical parameters as well as the stage according to Delacourte et al. (2002) are indicated.

Cases	Stage	Age	Clin Diagnosis	3D6 (1-42)(pg/ml)	Rb470 (8-42)(1/20)*
Duc	S0	26	Control	<125	0.073
Cru	S0	43	Control	<125	0.55
Rou2	S1	78	Control	<125	0.189
Pet	S1	83	Control	>10000	0.751
Cro	S2	72	Control	<125	0.05
Ben	S2	89	Control	<125	0.263
Mag	S2	95	Control	>10000	0.574
Fra	S10	64	Prob AD	>10000	1.225

* OD value of a 1/20 dilution of the formic acid extract after evaporation and solubilization in PBS.

Table 7. Summary of the demographic and CSF data of the patient groups clinically diagnosed with different neurological diseases that were analyzed in Example 3.

Group	n	Age (med; min-max)	MMSE (med; min-max)	Tau (pg/ml) (Avg, SD)	Phospho-tau (pg/ml) (Avg, SD)	Aβ1-42 (pg/ml) (Avg, SD)
Control	29	66 (61-80)	30 (28-30)	356 (152)	57 (23)	711 (164)
Mild AD	22	77 (68-87)	26.5 (24-28)	701 (216)	98 (42)	382 (80)
Mod AD	22	78.5 (49-89)	20.5 (17-23)	748 (234)	95 (36)	361 (115)
Sev AD	22	76.5 (61-84)	13.5 (2-16)	819 (359)	105 (46)	367 (61)
DLB	12	77 (65-87)	23 (17-27)	388 (134)	57 (15)	466 (34)
MCI-AD	14	78 (65-78)	29 (28-30)	654 (191)	97 (30)	503 (54)
Cogn	25	63 (39-92)	30 (25-30)	257 (126)	44 (18)	614 (163)
PD	15	71 (59-82)	29 (25-30)	306 (65)	54 (10)	671 (142)

Table 8. Molecular mass of the peaks observed on a PS-20 chip from Ciphergen coated with the carboxy-terminal 42 specific antibody 4D7A3. Experiments were done in duplicate on 100 µl of CSF.

Aβ sequence		11-42	10-42ox	8-42ox	5-42ox
Expected Mr		3335.92	3515.1	3659.23	4067.64
Group	Nr				
Control	150	-/-	3515.9/-	-/-	-/-
	148	-/3335.7	3514.6/3514.9	3653.2/-	-/-
	147	-/-	-/-	-/-	-/-
Cogn	87	-/-	3515.1/3516.1	-/-	-/-
	78	3337.2/-	3518.6/3519.4	-/-	-/-
	69	-/-	3516.8/3516.6	-/-	-/-
MCI-AD	111	-/-	3515.6/-	3652.4/-	-/-
	110	-/-	3515.3/-	3651.9/-	-/-
	112	-/-	-/-	-/-	4073.2/4071.8
Mild AD	54	-/-	-/-	-/-	-/-
	57	-/-	-/-	3652.6/3651.8	4072.2/-
	64	-/-	-/-	3652.5/3654.5	-/-
Mod AD	40	-/-	-/-	3653.4/3652.8	-/4074.4
	47	-/-	3516.5/3515.5	3654.7/-	-/-
	15	-/-	-/-	3652.8/3653.7	-/4071.6
Sev AD	31	-/-	3516.3/3523.5	3652.3/3652.7	-/4072.4
	32	-/-	-/3515.5	-/3652.1	-/4071.9
	22	-/-	3516.0/3523.2	3653.8/3653.3	-/4072.4
DLB	94	-/-	-/-	-/-	-/-
	101	-/-	-/-	-/-	-/-
	103	-/-	3517.9/-	-/-	-/-

The latter peptides are also observed in large quantities in the cerebral vessel walls, to constitute amyloid angiopathy, which is found in variable amounts in AD brains (Barelli et al., 1997; Wisniewski et al., 1997). Senile plaques consist largely of insoluble Aβ surrounded by a variety of neuronal and glial processes. This amorphous, acellular material is found in the spaces between the brain's nerve cells.

Both tau pathology and Aβ aggregation should be used as neuropathological diagnostic criteria for AD (Hyman and Trojanowski, 1997). Clinical AD is diagnosed in patients with tau pathology in the frontal pole and the parietal cortex (stages 7 to 10 according to Delacourte et al., 1999) and the presence of $A\beta_{42}$ aggregates above 50 µg/gram of wet tissue in these corical areas. Infraclinical AD is diagnosed in non-demented patients or patients with mild cognitive impairment, with insoluble $A\beta_{42}$ at a concentration of 10 µg/gram of tissue in neocortical areas, such as the frontal pole or the parietal cortex, and a tau pathology in the hippocampal area. In infraclinical AD patients, tau pathology can be asymptomatic up to stage 6. Non-demented patients can be considered as "pure controls" or "normal aging" (as far as tau and APP pathologies are concerned) if tau pathology is absent in all cortical areas, including the hippocampal area and if there is no trace of $A\beta_{42}$ aggregates in neocortical areas. If the patients are older than 75 years, very discrete or moderate tau pathology is likely to be found in the hippocampal area (stages 1 to 3), due to aging or a pathological process that remains to be determined. But these aged non-demented patients can be considered as controls as they have no detectable $A\beta_{42}$ aggregates (Delacourte et al., 1999; 2001).

A very significant effort is underway to test a large number of therapeutic options for AD. These approaches include numerous agents such as acetycholinesterase inhibitors, nonsteroidal anti-inflammatory drugs (NSAIDS), estrogen, neurotrophic agents, and even vitamins (Sramek and Cutler, 2000; Thal, 2000). Three general sub-approaches have arisen with the ultimate goal of limiting the presence of Aβ, in order to slow, stop or reverse the progression of AD. These approaches aim at preventing the formation of deposition of Aβ and to clear the already formed Aβ. The first way may be undertaken by enhancing the activity of α-secretase or inhibiting β- and γ-secretases. Upregulation or inhibition of enzymes is, however, a delicate and

APPENDIX 3

Background: The presence of amino-terminally truncated beta-amyloid (Aβ) species in plaques has been known for over two decades, but its potential relevance to the natural history of Alzheimer's disease (AD), has only recently been explored.

Materials and methods

Brain tissue samples (100 mg) from the Brain bank [1;2] were homogenized in 10 mM Tris-HCl pH 8.0 (1/10 wt/vol) containing a cocktail of protease inhibitors (Complete EDTA-free Protease inhibitors tablets: Roche Molecular Biochemicals, Basel, Switzerland), centrifuged for 1 hr at 4°C and the pellet was resuspended in 4 M GuHCl-TrisHCl pH 8.0.

The profile of Aβ42 species were analyzed using immuno-affinity enrichment with a C-terminal specific $A\beta_{42}$ specific antibody, 4D7A3 (Innogenetics) bound on a PS10 ProteinChip array and spectra were recorded using a SELDI-TOF (Ciphergen®).

Results:

Amino-terminally truncated Aβ species were abundantly present (see Figure 1 for an Aβ profile), not only in all clinically defined AD cases (n=9) but also in the preclinical stages (mild cognitive impairment (MCI) n=6; control n=24), in brain regions with the earliest neuropathologically detectable signs of AD. Amino-terminally truncated Aβ species were absent in 11 control subjects with an age of 42 or above and two younger controls. There are no obvious qualitative difference in amino-terminally truncated Aβ in AD or in DLB (n=9, Table 1).

Discussions and conclusions

$A\beta_{4-42}$, $A\beta_{8-42}$ and $A\beta_{1-42}$ appear before other N-terminally truncated Aβ42 peptides in brains of preclinical stages of AD

Reference List

1. A. Delacourte et al., Neurology 59, 398-407 (2002).
2. V. Deramecourt et al., J Neuropathol.Exp.Neurol. 65, 278-288 (2006).

Table 1: Profile of $A\beta_{42}$ peptides in 58 brain samples, biochemical and immunohistochemical characterized for Alzheimer pathology.

Clinical Diagnosis	Age	Cortical Biochemical Beta-amyloid staging	Cortical Biochemical PHF-Tau staging	Aβ11-42	Aβ10-42	Aβ8-42	Aβ7-42	Aβ6-42	Aβ5-42	Aβ4-42	Aβp3-42	A β2-42	A β1-42
	19	0											
	26	0	0										
	42	0	3										
	55	0											
	60	0	3										
	65	0											
	66	0											
	68	0											
	72	0											
	78	0											
	79	0	7										
	82	0											
	89	0											
Control	42	0	3										
Control	65	0	4										
Control	72	0	2										
Vascular dementia	78	0	3										
Control	89	0	2										
PD	48	0	7										
Vascular dementia	75	0	7										
Control/ MCI	85	0											
	72	0											
	95	0	4										
encephalitis	44	0	3										
control	60	0	3										
MCI/ vascular	66	0											
leuco encephalitis	36	0	0										
control	43	0	0										
Control	76	1											
control	82	1	3										
MCI	83	1											
Vascular dementia	79	1	7										
PD	69	1											
Encephalopat hy	76	2											
Pneumopath y	60	3	4										
Control	85	4	2										
MCI	86	4	3										
Control	77	5	3										
MCI	83	6	1										
MCI	72	7	3										
MCI	84	7	3										
AD	73	7	10										
FAD	63	7	10										
FAD	69	7	10										
FAD	51	8	9										
AD	73	8	10										
AD	80	8	10										
FAD	67	8	10										
AD	54	10	10										
DLB	76	0	5										
DLB	77	4	7										
DLB	82	6	7										
DLB	80	7	5										
DLB	64	8	7										
DLB	61	8	10										
DLB	65	8	10										
DLB	82	9	5										
DLB	71	9	10										